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(54) Title: A NOVEL CONJUGATE, ITS PREPARATION AND USE AND A SUBSTRATE PREPARED WITH THE CONJUGATE

(57) Abstract

The invention relates to a substantially water-soluble biologically active conjugate comprising a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, via which groups a number of molecules from the group of sulphated glycosaminoglycans in a non-active part thereof are anchored through covalent bonds. The invention also relates to the production of the conjugate, the preparation of a substrate surface with the conjugate, a substrate surface thus prepared, and the conjugate for use as a therapeutical agent.

A NOVEL CONJUGATE, ITS PREPARATION AND USE AND A SUBSTRATE
PREPARED WITH THE CONJUGATE

The present invention relates to a novel biologically active conjugate based upon sulphated glycosaminoglycan, a process of preparing the conjugate, a substrate whose surface has been prepared with such a conjugate, and a process of surface-preparation using the conjugate.

Sulphated glycosaminoglycans is the common name of a number of endogenous sulphated mucopolysaccharides, such as e.g. heparin, heparan sulphate, dermatan sulphate and chondroitin sulphate, which exhibit a number of different biological properties. While the invention relates to sulphated glycosaminoglycans generally, it will, however, in the following to a great extent be described with regard to the glycosaminoglycan that has so far found the greatest medical use, viz. heparin.

Heparin occurs naturally complex-bound to protein in various mammalian tissues, such as the intestine, liver and lung, as well as in mast cells, and has then a molecular weight which may extend up to 100,000, while commercially available preparations have a molecular weight varying between about 6,000 and 20,000 depending on the source and the determination method. It consists of alternating glucuronic acid and glucosamine units, and the anti-coagulating effect has been shown to be linked to a specific pentasaccharide unit of the molecule which has antithrombin-binding properties.

Due to its anti-coagulating properties, heparin, which is usually prepared from intestinal mucosa from pig, has found use as an agent for dissolving thromboses, but perhaps above all for preventing the formation thereof. The latter is the case for inter alia procedures in, for example, the treatment of renal disease, open cardiac surgery and intensive care, which procedures involve treating the patient's blood in a circulating system outside the body, so-called extra-corporeal circulation (e.g. artificial kidneys, heart-lung machines,

oxygenators), where the blood will get in contact with
various materials foreign to the body.

To eliminate the clotting ability of the blood in such
systems, and thereby avoid clogging by blood clots, high
5   doses of heparin must be added to the blood. Due to the
accompanying substantially increased risk of bleeding,
which in the worst case may lead to life-threatening
conditions, efforts have for a long time past aimed at
trying to instead achieve the desired coagulation
10   preventing effect by modifying the body-foreign material
with which the blood will get in contact by surface-binding
heparin thereto. Decisive factors which have stimulated
this development are that the structure-activity relation
of heparin has been elucidated, and that a heparin-like
15   activity has been detected on the natural vascular wall.
Thus, during the last few years several reports on
successful extra-corporeal treatment with systems provided
with surface-bound heparin have been published.

The surface-modification with heparin has, however,
20   not been restricted the above mentioned contexts with
extra-corporeal blood circulation, but has also come to be
regarded as a more general solution to the problem of
achieving biocompatibility of various devices within
medical care which get in contact with blood and other body
25   tissues. For example, surface-heparinization has also been
used to improve the biocompatibility of intraocular lenses.

The hitherto used technical solutions to the problem
of immobilizing heparin may be divided into two main
principles, ionically and covalently bound heparin, which
30   will be described in more detail in the following. To
accomplish a surface which exhibits the desired
biocompatibility based on immobilized heparin, it is
important that heparin is immobilized such that its
biological activity is maintained. As mentioned in the
35   introductory part, the biological activity of heparin
resides in a specific antithrombin-binding pentasaccharide
structure which must remain intact after the immobilization
on the surface to be capable of interacting with the con-

stituents of the blood. In the majority of scientific
articles and patents related to immobilization of heparin,
in particular those published earlier than 1980, this
aspect has not been satisfied, and much less have results
5   been presented which makes it possible to judge whether the
preparation method will lead to a perfect biocompatible
surface. Hereinafter a review of previously known methods
of immobilizing heparin is given.

                    I. IONICALLY BOUND HEPARIN
10   Since heparin contains a great number of negatively
charged groups, the heparin molecule is capable of binding
relatively strongly to cationic surfaces through only
electrostatic interaction. A common procedure consists in
precipitating heparin from an aqueous solution thereof with
15 a cationic surfactant, and subsequently dissolving the
dried precipitate with an organic solvent. The latter
solvent is then used for a so-called dip-dry procedure.
Various branched surfactants have been tested in order to
reduce the release rate. Other methods are based upon
20 adsorption of heparin to quaternary ammonium groups. A
great disadvantage which the ionic bound heparin surfaces
have in common is that their stability with regard to the
release of heparin in contact with blood is insufficient.
            O. Larm et al. describes in Biomat., Med. Dev., Art.
25 Org., 11 (1983) 161-173 inter alia a method of preparing a
stable ionically bound surface. The bound heparin is,
however, reported to have lost its biological activity,
which may be related to the fact that each individual
heparin molecule was bound too strongly such that the anti-
30 thrombin binding sequence could not interact with
circulating components in blood.
            A stabilizing treatment of an ionically bound heparin
complex with glutaraldehyde has been described in US-A-
3,810,781 and US-A-4,118,485. As appears from scientific
35 reports, these preparation alternatives do not lead to
completely stable surfaces. Thus, heparin and probably
various reaction products with glutaraldehyde are released
to the blood path during the initial contact phase.

II. COVALENTLY BOUND HEPARIN

From a purely chemical viewpoint there are a number of different ways of immobilizing heparin with covalent bonds. With cyanogen bromide, carbodiimide and similar generally used coupling reagents there is, however, an obvious risk that each heparin molecule will be bound by several bonds including bonds in the active sequence, and that the heparin thereby will lose its biological activity. Covalent coupling reagents are besides always toxic as such and should therefore not get into contact with the final product.

US-A-4,613,665 describes, however, a method of coupling heparin and other polysaccharides via a single reactive aldehyde group located terminally in the heparin molecule. Hereby heparin may be bound covalently without the antithrombin-binding sequence being involved in coupling. However, the method requires that heparin is partially degraded and that the strongly toxic substance cyanoborohydride is present in the final preparation step.

EP-A-351,314 describes a method of coupling heparin to substrate surfaces containing free amino groups (through treatment of the surface with e.g. polyethylenimine or chitosan) by utilizing free amino groups of heparin which have been subjected to N-desulphation. Cross-linking is then performed with polyfunctional aldehydes, such as e.g. glutaraldehyde. The reaction step with glutaraldehyde can, however, not be controlled with certainty to prevent the active sequence from being involved, and the method as such is relatively complicated to carry out from the technical point of view.

US-A-4,239,664 describes a PVP-heparin polymer prepared through modification of PVP such that the polymer contains imidoyl ions which then are reacted with hydroxyl groups on heparin. The method necessarily gives multiple non-specific bonds to the heparin, detrimentally effecting the biological activity thereof. The PVP-heparin polymer is consistently said to have a low anti-coagulating activity.

EP-A-294 905 discloses a polymer substrate having an
anticoagulant like heparin linked thereto via a polyacid.
The substrate is prepared by covalently binding the
polyacid to a small number of reactive groups on the
polymer surface to thereby multiply the number of available
surface reactive groups. The anticoagulant is then
covalently coupled to carboxyl or amino groups of the
polyacid, specifically by the method described in the
above-mentioned US-A-4,613,665, the disadvantages of which
has already been mentioned.

US-A-4,415,490 discloses a non-thrombogenic material
wherein heparin is coupled to various polymers through only
one acetal or hemiacetal bond at each binding site. In one
embodiment, aldehyde groups are introduced into a polymer,
such as cellulose, which aldehyde groups then are reacted
with hydroxyl groups in heparin. This process will involve
a plurality of the hydroxyl groups of each heparin
molecule, and since hydroxyl groups are available in the
biologically active sequence of heparin, which sequence was
not actually known and described in the literature on the
filing date of the patent, there is an apparent risk that
also hydroxyl groups in the active sequence will be
involved, resulting in the final product being inactive. In
an alternative embodiment, aldehyde groups are instead
introduced into the heparin by periodation. Also this
embodiment lacks specificity, and binding will therefore
occur randomly in the heparin chain, including the active
sequence.

As will appear from the above, the methods known so
far for surface-heparinization thus suffer from more or
less serious disadvantages. There is therefore a need for a
method of surface-heparinization which is simple to perform
and which provides a stable heparinized surface free from
toxic substances and with retained biological activity of
the heparin.

Also the use of heparin as a therapeutical agent has
limitations due to the short half-life and/or affinity of
heparin. This is particularly the case for the use of

heparin as an anticoagulant, but also, for example, its studied use as a growth inhibitor of smooth muscle cells in case of vessel damages (hyperplasia), as an anti-inflammatory agent for e.g. rheumatoid arthritis, and as an agent for controlling the formation of blood vessels (angiogenesis). A review of the different properties of heparin is to be found in "Heparin: Clinical and biological properties. Clinical applications." Eds: Lane and Lindahl, Edward Arnold, London, 1989. There is therefore a need of heparin preparations having a prolonged half-life and increased affinity.

According to the present invention a novel biologically active conjugate is suggested based upon sulphated glycosaminoglycans, by means of which conjugate the properties of the sulphated glycosaminoglycans may be utilized considerably more efficiently than with the individual substances. Such a conjugate may inter alia easily be made to bind stably to a substrate surface having affinity to the conjugate and may thereby, for example, in the case of heparin be used for simpler and more efficient surface-heparinization than with previous methods. Further, such a conjugate may provide glycosaminoglycan preparations having a longer half-life and an improved affinity compared with preparations based upon the pure substances.

As already mentioned for heparin, the sulphated glycosaminoglycans exist naturally bound to proteins. Thus, for example, in the case of heparin, about 15 heparin chains are bound to a protein of about 25 amino acid residues, while a proteoglycan containing heparan sulphate has fewer and considerably more sparsely arranged heparan sulphate chains. The natural conjugates are very difficult to prepare in pure form and have as far as we know not been suggested for therapeutical or similar use. The invention is based upon the concept of providing a semi- or fully-synthetic conjugate between sulphated glycosaminoglycans and a polymeric carrier, which conjugate, inter alia by containing more molecules of the glycosaminoglycan in question, has improved properties in relation to the

individual glycosaminoglycans as well as the natural
conjugates, and which conjugate further has the important
advantage that the relative composition may be varied in a
controllable way to suit different applications.

In its broadest scope the present invention thus
provides an at least substantially water-soluble,
biologically active conjugate (macromolecule), preferably
in substantially pure form, comprising a substantially
straight-chained organic homo- or heteropolymer having a
number of functional groups distributed along the polymer
backbone chain, via which groups at least about 20
molecules from the group of sulphated glycosaminoglycans
(GAG) in a non-active part thereof are anchored through
covalent bonds.

Such a conjugate may conceptually be described as a
synthetic proteoglycan, the relative composition of which
may be varied in a controllable way and adapted to the
intended application.

The expression "sulphated glycosaminoglycans" herein
is meant to comprise not only the substances which are
normally included in the term, such as e.g. heparin,
heparan sulphate, dermatan sulphate and chondroitin
sulphate, but also fragments and derivatives of these
substances which are functional for the purpose.

The substantially linear polymer chain which is to
function as the carrier for the glycosaminoglycan residues
should, of course, be substantially biologically inert
after the coupling of the glycosaminoglycan or -glycans in
question, in the sense that it should be devoid of at least
interfering biological activity. As is readily understood,
it should in order to permit coupling of a plurality of
glycosaminoglycan residues be provided with a number of
functional groups, such as e.g. amino, hydroxyl or carboxyl
groups, distributed along the chain and capable of, after
optional modification, being coupled to the
glycosaminoglycan, either directly or via a coupling
sequence. It is in this context to be noted that the
glycosaminoglycan in question, depending on its

preparation, may still have the terminal residue of its natural conjugate protein associated thereto, and that the binding then, of course, advantageously will take place via e.g. an amino acid in such a residue.

5   Further, the carrier polymer should preferably have a good solubility in water. At least it should, in accordance with what has previously been said about the conjugate, be at least substantially water-soluble after the coupling of the glycosaminoglycan groups. Specific polymer chains which

10   may be suitable for the purposes of the invention will readily be apparent to the skilled person after having taken part of the general inventive concept. This is, of course, also the case for the degree of branching on the polymer chain that may be permitted within the scope of the

15   expression "substantially linear".

Preferably, however, the polymer chain is a natural or synthetic polypeptide, polysaccharide or aliphatic polymer. As specific examples may be mentioned polylysine, polyorni-thine, chitosan, polyimine and polyallylamine.

20   With regard to the fact that it is usually desired that the glycosaminoglycan will maintain its biological activity after the binding to the polymer carrier, it is preferred that each glycosaminoglycan molecule is bound terminally and by only a single bond to the carrier

25   polymer. Suitably, the glycosaminoglycan is bound via an amino acid, and then preferably a terminal amino acid, but also free amino groups of a glucosamine unit may be used. The latter may exist free as such or may have been liberated through desulphation or deacetylation.

30   The number of glycosaminoglycan residues per polymer backbone chain is, as mentioned above, at least 20, but preferably higher, usually at least 30. Depending on the polymer backbone chain used, it may be preferred to have at least 60 and even more than 100 glycosaminoglycan residues

35   per polymer backbone chain, as will appear from the working examples presented further on. The upper limit depends on the circumstances and is set inter alia by the solubility properties of the selected carrier polymer, how high a

viscosity that may be permitted, etc. In addition to the
intended use of the specific conjugate, the optimum number
of glycosaminoglycan units will also depend on the carrier
polymer, and then particularly the size thereof. In the
5   case of electrostatic binding of the conjugate to a
substrate surface, which will be discussed in more detail
below, the charge density of the substrate surface will, of
course, also have to be considered. Thus, the glycosamino-
glycan residues should not be located so closely that they
10 will interfere with each other, but neither should they
have too wide gaps between them. As an example it may be
mentioned that e.g. polylysine as a carrier polymer should
have a molecular weight higher than about 50,000. The
suitable number of glycosaminoglycan residues for each
15 specific carrier polymer and use, respectively, will,
however, readily be determined by the skilled person.
        Particularly in the case that an amino-functional
polymer is used as the carrier, it may in some cases,
especially when the polymer backbone chain is sparsely
20 substituted with glycosaminoglycans, be favourable to block
the remaining free amino groups, which, for example, may be
effected by acetylation. An alternative approach might be
to substitute a desired number of amino groups with e.g.
methyl groups before attaching the glycosaminoglycans.
25   As already indicated, the novel conjugate according to
the invention may be bound to a surface having affinity to
the conjugate (usually but not necessarily to the glycos-
aminoglycan residues) so as to thereby provide the surface
with the desired biological activity. According to a
30 further aspect of the invention, such a prepared surface is
accomplished by simply contacting, under suitable
conditions, a biologically active conjugate comprising a
substantially straight-chained organic polymer having a
number of functional groups distributed along the polymer
35 backbone chain, via which groups a number of molecules from
the group of sulphated glycosaminoglycans are anchored by
covalent bonds, with a surface having affinity to the
conjugate.

Another aspect of the invention provides a substrate surface having affinity-bound thereto a biologically active conjugate comprising a substantially straight-chained organic polymer having a number of functional groups distributed along the polymer backbone chain, via which groups a number of molecules from the group of sulphated glycosaminoglycans are anchored by covalent bonds.

A preferred form of affinity between the conjugate and the substrate surface is of electrostatic nature, and more particularly that binding takes place by electrostatic interaction between the glycosaminoglycan residues and the substrate surface, as will be illustrated in more detail hereinafter.

Since the glycosaminoglycan molecules of a conjugate according to the invention are in great excess in relation to the carrier polymer, the conjugate may be regarded as a "macromolecular glycosaminoglycan". The number of anion groups per conjugate will thereby by far exceed the number existing per glycosaminoglycan molecule, which results in the conjugate, by virtue of its size, being capable of binding irreversibly to a cationic surface through ionic interaction. For the conjugate to be released from the surface, all glycosaminoglycan residues must, of course, be released from the surface simultaneously, which in contrast to the release of "free" glycosaminoglycan molecules will require a considerable supply of energy.

With the exception of certain situations which will be described further on, it is generally intended that the biological activity of the conjugate should be due to the glycosaminoglycan residues. In such a case, the number of glycosaminoglycan residues should be sufficient for a certain part of these residues per carrier polymer chain to be capable of together mediating a strong, irreversible binding to a surface which has been provided with cationic groups, while the remaining glycosaminoglycan chains freely can exert their biological activity by interacting with a biological tissue, e.g. the constituents of the blood.

Surface-preparation with a glycosaminoglycan according to the above is thus based upon a combination of covalent binding and ionic interaction, a considerable advantage being that the conjugate is prepared as an intermediate product, which means that all coupling chemistry may be made separately from the final product. Further, the final surface modification process becomes very simple and may be performed in a reproducible manner. For example, surface-heparinization with a heparin conjugate according to the invention therefore provides, as already mentioned, a considerably simplified method for efficient heparinization in relation to current methods for surface-heparinization. Notwithstanding the foregoing, it is, of course, possible to optionally carry out a cross-linking step after the affinity adsorption of the conjugate to the substrate surface in order to improve the stability of the heparinized surface even further.

A conjugate for use according to this particular aspect of the invention will thus have an electrostatic net charge which is sufficient to permit substantially irreversible binding to an oppositely charged substrate surface.

The substrate material to be surface-prepared, e.g. surface-heparinized, according to the above may in principle be any material that is desired to be made biocompatible, provided that its surface is or may be made cationic. As described previously, the invention may apply to a body-foreign material, such as various polymers, metals and ceramics. It may, however, also apply to an endogenous material, i.e. a tissue surface exhibiting affinity for the glycosaminoglycan in question. In this connection it is of interest to note that the healthy natural vessel wall in its outermost structure against the blood contains sulphated glycosaminoglycans having antithrombin-binding pentasaccharide sequences.

Various methods for making a substrate surface cationic are well known. Treatment with polyimine has proved to be a suitable method, but also other polyamines,

such as e.g. polylysine, chitosan or polyallylamine, may be
used, as will be described in the working examples below.

The novel glycosaminoglycan conjugate may within the
scope of the invention also contain chains of one or more
5   other substances bound to the carrier polymer in addition
to the glycosaminoglycan chains, e.g. another biologically
active substance. Such other biologically active substance
may in that case be intended to act simultaneously with or
separate from the glycosaminoglycan activity. In the latter
10 case, only the biological activity of the complementary
substance would be of interest, the glycosaminoglycans only
being utilized for the affinity binding to the substrate
surface. The conjugate according to the invention may thus
also only function as a carrier for desired biologically
15 active substances which one wants to bind to a surface.
Examples of substances which in addition to the
glycosaminoglycans may be bound to the polymer backbone
chain are growth factors, enzymes, antibodies, matrix
proteins, steroids, etc. It is in this context also to be
20 noted that a conjugate with very specific adsorption
properties may be accomplished, for example, with
monoclonal antibodies as a complement to the
glycosaminoglycan units.

Optionally, it may in the case of such combined
25 conjugates be desired to suppress the biological activity
of the glycosaminoglycan itself, which, for example, in the
case of the coagulation inhibiting activity of heparin may
be effected by desulphation. In such a case, the biological
activity of the conjugate would thus be completely linked
30 to the activity of complementary substances which are bound
to the polymer backbone chain.

While in many cases it is the surface-binding effect
of the conjugate that is essential, not to say necessary,
this effect is, however, in certain cases of less interest,
35 and it may even for some applications be desired to
suppress it more or less completely. In the same way it
may, as mentioned above for the combined conjugates, also
in the case of a pure glycosaminoglycan conjugate be

desired to eliminate or at least reduce the biological
activity of the glycosaminoglycans. In some cases, such as
e.g. for heparin, the glycosaminoglycan may have several
different biological effects, and depending on the intended
5   application, one biological activity may then be suppressed
in favour of another. For example, in the case of heparin
the anticoagulating effect thereof may as above be
inhibited by desulphation, while another biological
activity which is not linked to the previously mentioned
10 pentasaccharide unit will remain unaffected.

As appears from the above, the composition of the
novel conjugate may thus be varied within wide limits to be
adapted to different application fields.

Another aspect of the invention relates to the
15 production of the described conjugate by providing a
substantially straight-chained organic polymer having a
number of functional groups distributed along the polymer
backbone chain, and to these functional groups, optionally
via a coupling reagent, covalently binding a number of
20 molecules from the group of sulphated glycosaminoglycans in
a non-active part thereof. This may within the scope of the
invention be performed in several different ways.

Thus, the glycosaminoglycan may, for example, be bound
directly to an amino-functional polymer chain utilizing a
25 nitrous acid degraded glycosaminoglycan having a terminally
located aldehyde group prepared according to the method
described in US-A-4,613,665. This method involves, however,
a restriction to a partially degraded glycosaminoglycan,
and the degree of substitution is difficult to control.
30 Also, practical difficulties arise due to the fact that the
polymer is easily precipitated by the glycosaminoglycan.

According to a preferred method, the glycosaminoglycan
is instead bound to the polymer chain by means of a
coupling reagent, and preferably a heterobifunctional one.
35 It may be noted that bifunctional coupling reagents for
e.g. hydroxyl or amino groups can, however, generally not
be used, since they will lead to intra- as well as inter-

molecular cross-linking with consequential blocking and
aggregation, respectively.

As an example of how a conjugate according to the
invention may be prepared, the coupling of heparin to a
5   polylysine will now briefly be described. By selecting a
polylysine having a molecular weight above 400,000, a
synthetic proteoglycan having up to 500 heparin chains per
carrier molecule may be prepared. A hetero-bifunctional
coupling reagent suitable for this purpose, N-succinimidyl-
10   3-(2-pyridyldithio)-propionate (SPDP), is coupled to amino
groups on the polylysine, the SPDP-substituted polylysine
then being purified chromatographically. In a separate
coupling step, SPDP is also coupled to amino groups on
heparin which are present either in terminal amino acid
15   residues or as free glucosamine (the latter content may be
controlled via N-desulphation or N-deacetylation). The
SPDP-groups are reduced to thiol function, whereupon the
SH-substituted heparin is purified chromatographically. The
content of SPDP groups in polylysine and SH-groups in
20   heparin, respectively, are determined spectro-
photometrically, and heparin is mixed with polylysine in an
equimolecular amount with regard to SPDP and SH, heparin
being bound covalently to polylysine via disulphide
exchange, the reaction rate of which may be followed
25   spectrophotometrically. It has surprisingly been found that
the precipitation reaction between polylysine and heparin
does not take place when polylysine has been provided with
SPDP-groups, even if only a certain portion of the amino
groups of polylysine have been substituted. Nevertheless,
30   practical experiments have shown that the disulphide
exchange is quicker and proceeds to completion only at a
high salt concentration (suitably 3 M NaCl). When the
reaction is completed, the conjugate is purified
chromatographically, free heparin and low-molecular
35   reaction products being removed.

Concerning the stability of the thus prepared heparin
conjugate in different environments, it has surprisingly
been found that the obtained disulphide bridges which

couple heparin to the polymer backbone chain cannot be
cleaved with glutathione but only with low-molecular non-
physiological thiol reagents, such as e.g. mercaptoethanol.

It is further to be noted that a substantial advantage
of heparinizing with a heparin conjugate according to the
invention resides in the possibility of starting from a
less processed heparin raw material than in the
conventional processes.

The invention is illustrated further in the following
Examples.

EXAMPLE 1

Preparation of conjugates and test of surface-bound biological activity

Two different batches of heparin (Heparin, Kabi
Pharmacia AB, Sweden, molecular weight about 12,000) was
used. The content of amino acids and the relative
occurrence of free primary amino groups were analysed, the
following results being obtained.

	Amino acid nitrogen (μg/ml)	Total nitrogen (μg/ml)	Primary amine (rel. scale)
Heparin A	0.36	5.38	5,000
Heparin B	0.08	5.37	340

Since heparin B exhibited a very low content of free
amine, N-desulphation according to the method described by
Yuko Inone et al., Carbohydrate Research, 46 (1976) 87-95,
was performed. After carried out N-desulphation, the value
18,000 on the relative scale for primary amine was
obtained.

Heparin A and heparin B (desulphated) were dissolved
in phosphate buffer, pH 7.5, (200 mg/4 ml), to which 1 ml
of SPDP (10 mg/ml MeOH) was added under stirring, and the
reaction was allowed to proceed for 20 minutes. The SPDP-
substituted heparin thus obtained was purified on Sephadex®
G-25 (Pharmacia LKB Biotechnology AB, Sweden). To 100 μl of
the obtained sample were added 900 μl of dithiothreitol
(DTT, 10 mg/ml), and the obtained absorbance was measured
spectrophotometrically at 343 nm. The substitution-degree

for heparin A was 0.21 and for heparin B (desulph.) 0.17.
SPDP coupled to heparin was reduced to SH by the addition
of DTT and subsequent chromatographical purification.

5   Polylysine having a molecular weight of 450,000 was
dissolved in water (20 mg/3 ml), to which 2 ml of SPDP
(10 mg/ml MeOH) were added, and the reaction was allowed to
proceed under shaking for 20 minutes. Purification was
performed on Sephadex® G-25 (Pharmacia LKB
Biotechnology AB, Sweden) with 0.15 M NaCl as eluent. The

10   void fraction was tested with DTT, the substitution-degree
being determined as 158 SPDP-groups per molecule of
polylysine.

The above prepared solutions of heparin-SH and
polylysine-SPDP, respectively, were adjusted to 3 M NaCl

15   and mixed in such proportions that a ten percent excess of
SH-groups in relation to SPDP-groups was obtained, and the
reaction was allowed to proceed overnight. Both
preparations (heparin A and heparin B (desulph.)) had then
proceeded to completion, which was determined by

20   spectrophotometrical measurement of the release of
thiopyridone at 343 nm. The preparations were purified on
Sephacryl® S-500 (Pharmacia LKB Biotechnology AB, Sweden)
with 0.5 M NaCl as eluent, the heparin-polylysine
conjugates emerging as a void peak with baseline separation

25   to free heparin. The content of heparin was determined with
the Orcinol assay described in Larsson, R., et al.,
Biomaterials 10 (1989) 511-516.

The respective heparin conjugates were then diluted to
50 μg heparin/ml in citrate buffer, pH 3.8, with addition

30   of 0.5 M NaCl. Tubings of polyethylene (PE) were surface-
heparinized by treatment as follows:

1)   Ammonium persulphate (1 %, 60°C, 120 min.)
2)   Polyethylene imine (0.3 mg/ml, RT, 15 min.)
3)   Heparin conjugate solution as above (RT, 120 min.).

35   The tubings were finally flushed with borate buffer, pH 9,
2 x 10 min., and water.

The surface-heparinized tubings were tested with
regard to the inhibiting capacity of thrombin according to

the following method. The tubings were first rotated with
human plasma during 90 minutes, whereupon they were rinsed
with sodium chloride solution. Then the tubings were
incubated with a solution of thrombin (15 U/ml, 10 min.,

5   RT, under rotation) and rinsed with sodium chloride
solution. Half of the tubings were then incubated with
defibrinogenated plasma for 60 seconds. Surface-bound
thrombin activity was measured by incubating the tubings
with a chromogenic substrate for thrombin during 60

10 seconds, after which the reaction was stopped by the
addition of citric acid. The obtained absorbance was
measured at 405 nm. The following values were obtained.

	Conjugate with Heparin A	Conjugate with Heparin B (desulph.)
15 Uptake of thrombin (without defib. plasma)	0.639 ± 0.050	0.611 ± 0.156
Residual amount of thrombin (with 20 defib. plasma)	0.003 ± 0.001	0.006 ± 0.001

The results indicate that both preparations provided a
perfectly satisfactory effect with regard to uptake and
inhibition of thrombin.

EXAMPLE 2

25 **Preparation of conjugates with different substitution-
degrees and test of surface-bound biological activity**

A conjugate, called Conjugate I, was prepared as
described in Example 1. The final substitution-degree of
heparin per polylysine was 240:1.

30 Then another conjugate, called Conjugate II, was
prepared. In this case the starting material was a
polylysine with a higher substitution-degree of SPDP,
prepared by adjustment of the pH to 8 in the polylysine
solution before the addition of SPDP. The substitution-

35 degree was determined as 633 SPDP-groups per polylysine
molecule.

Heparin-SH was prepared as in Example 1 and was
reacted with the highly substituted polylysine obtained

above. The reaction proceeded to 77 % conversion, and the substitution-degree of heparin per polylysine was therefore 490:1. Tubings of polyethylene (PE) were prepared and tested as described in Example 1, the following results being obtained.

	Conjugate I	Conjugate II
Uptake of thrombin (without defib. plasma)	0.516 ± 0.021	0.526 ± 0.031
Residual amount of thrombin (with defib. plasma)	0.011 ± 0.001	0.008 ± 0.001

The results indicate that both conjugates provided satisfactory results.

EXAMPLE 3

Test of surface-heparinized extra-corporeal system

An extra-corporeal system composed of the following components was used: Drainage catheter (polyvinyl chloride (PVC)), arterial cannula (PVC + steel), tubing set (PVC), pump bladder (ethylbutyl acrylate), valves (polypropylene (PP) + PE), oxygenator (polycarbonate + hollow-fibres of PP).

All the components were surface-heparinized by treatment in three steps:

1)   Ammonium persulphate (1 %, 60°C, 120 min.)

2)   Polyethylene imine (0.3 mg/ml, borate buffer, pH 9, RT, 15 min.)

3)   Heparin-polylysine conjugate, prepared according to Example 1, was diluted to 30 µg/ml in citrate buffer, pH 3.8, with 0.5 M NaCl, and was treated at room temperature for 120 minutes. The components were finally rinsed 2 x 15 min. with borate buffer, pH 9, and water. After drying, sterilization with ethylene oxide was carried out.

The extra-corporeal system was connected to an anaesthetized pig which had not received any anti-coagulant therapy for partial by-pass between the right atrium and the aorta. The external system continuously pumped about

three litres per minute during twenty-four hours without
any problems with clogging due to coagulation. The
coagulation time was all the time at a constant value,
indicating that there was no release of heparin to the
blood path.

The results demonstrate that a complete system for
extra-corporeal support circulation easily may be surface-
heparinized with the heparin conjugate to obtain a stable,
well-functioning heparin surface, which may be sterilized
with ethylene oxide.

EXAMPLE 4

Test of biological activity of various heparin-conjugates in solution

Heparin-polylysine conjugates with different
substitution-degrees were prepared according to Examples 1
and 2. The biological activity of the conjugates was
determined with regard to their capability of inhibiting
Factor Xa and thrombin in buffer solution containing anti-
thrombin or in plasma. The results obtained were compared
with corresponding standard graphs obtained by the addition
of known amounts of heparin having a known specific
biological activity (180 I.U./mg). The following results
were obtained.

<u>Biological activity I.U./mg heparin</u>

Conjugate	Heparin/ polylysine	Xa/AT	Xa/Plasma	Tr./AT	Tr./Plasma
I	235	116	95	48	45
II	490	61	29	10	20
III	550	10	43	18	25

The results indicate that the described process may be
utilized to prepare conjugates having a high as well as a
low biological activity.

EXAMPLE 5

Effect of different polylysine sizes

Five different batches of polylysine with the
respective molecular weights 13,000, 64,000, 98,000,
249,000 and 464,000 were modified with SPDP in accordance

with Example 1, the following substitution-degrees (SPDP-
groups per molecule of polylysine) being obtained:

Sample	Molecular weight	Substitution-degree
I	13,000	6
II	64,000	31
III	64,000	45
IV	98,000	35
V	249,000	87
VI	464,000	158

Heparin, having the value of 7,000 on the relative
scale for primary amine, was modified with SPDP for the
introduction of free thiol groups in accordance with
Example 1, substitution-degrees of 0.2 - 0.3 being
obtained. Respective conjugates were prepared according to
Example 1. Separation was performed on a column with
Sephacryl® S-300 or Sephacryl® S-400 (Pharmacia LKB
Biotechnology AB, Sweden) as the separation medium.
Conjugate I could not be separated from free heparin. For
the other conjugates satisfactory separation was obtained,
and the conjugates obtained could be used for surface-
heparinizing tubings according to Example 1. Testing with
regard to uptake and inhibition of thrombin (according to
Example 1) gave the following results:

Conjugate	Uptake of thrombin (without def. plasma)	Residual amount of thrombin (with def. plasma)
I	-------------	-----
II	0.012 ± 0.006	0
III	0.086 ± 0.047	0
IV	0.494 ± 0.009	0.003
V	0.532 ± 0.043	0.005
VI	0.490 ± 0.004	0.004

The results indicate that conjugates II - VI may be
used according to the invention to prepare surfaces with
heparin activity. However, conjugates IV - VI give the best
results.

EXAMPLE 6

Preparation of conjugate with chitosan as carrier substance

Chitosan (SeaCure 110L, viscosity <20 mPas, molecular weight about 120,000, Protan Biopolymer A/S, Drammen, Norway) was dissolved to 10 mg/ml in water with 1 % acetic acid. To 1.5 ml solution was added 1.0 ml SPDP (10 mg/ml MeOH) under stirring at 50°C, and the reaction was allowed to proceed for one hour. The sample was loaded on a PD-10 column (Pharmacia LKB Biotechnology AB, Sweden) and eluted with 0.5 M NaCl with 1 % acetic acid. The void fraction was collected and analysed for presence of SPDP. The content of SPDP was determined as 0.972 μmole/ml, corresponding to about 40 SPDP-groups per chitosan molecule.

Heparin was prepared with free thiol groups according to Example 1. To the obtained heparin solution was then added sodium chloride to a final concentration of 3.5 M. The heparin solution was then added to the first prepared chitosan-SPDP solution under vigorous stirring, and the reaction was allowed to proceed at room temperature overnight. Spectrophotometrical control indicated that the reaction had proceeded to 100 %. The solution was fractionated on Sephacryl® S-300 (Pharmacia LKB Biotechnology AB, Sweden) and the void fraction was collected. Tubings of Pebax® (polyether block amide from Atochemie, France) were prepared for thrombin test according to Example 1. The following results were obtained:

Uptake of thrombin (without def. plasma)	Residual amount of thrombin (with def. plasma)
0.491 ± 0.016	0.002

The results demonstrate that a surface prepared with a chitosan-heparin conjugate gives a perfectly satisfactory effect.

EXAMPLE 7

Preparation of conjugates with polyallylamine

Polyallylamine hydrochloride (Aldrich, molecular weight about 50,000), 10 mg, was dissolved in 1.5 ml of borate buffer, pH 9, to which 1.0 ml SPDP (10 mg/ml MeOH)

was added under stirring and reacted for 30 minutes. The
solution was loaded on a PD-10 column, which was eluted
with 0.9 % NaCl. The void fraction was collected, and
analysis indicated that it contained 8.46 µmole/ml SPDP,
5   corresponding to about 192 SPDP-groups per molecule of
polyallylamine. This product was used below for the
preparation of Conjugate I.

Another 10 mg of polyallylamine hydrochloride,
dissolved in water instead of borate buffer, were SPDP-
10   substituted in the same way as above. The void fraction
then contained 1.56 µmole SPDP/ml, corresponding to 32
SPDP-groups per molecule of polyallylamine. This product
was then used for the preparation of Conjugate II below.

Another preparation utilized polyallylamine
15   hydrochloride which had been partially methylated by
reacting 2 µmole of polyallylamine dissolved in 7 ml of
water with the pH adjusted to 3.5 with 861 µmole of
formaldehyde in the presence of 1610 µmole of
cyanoborohydride. After reaction over night, the modified
20   polyallylamine was purified on Sephadex® G-25 (Pharmacia
LKB Biotechnology AB, Sweden). Modified polyallylamine
hydrochloride, 10 mg, was dissolved in borate buffer, pH 8,
and was substituted with SPDP as described above. The void
fraction then contained 1.5 µmole/ml, corresponding to
25   about 50 SPDP groups per molecule of polyallylamine. This
product was then used for the preparation of Conjugate III
below.

Heparin was prepared with free thiol groups according
to Example 1, whereupon the resulting heparin-SH was mixed
30   with the respective polyallylamine-SPDP products in an
equimolecular relation with regard to SH- and SPDP-groups.
After reaction for one hour the salt content was raised to
3 M, and the reaction was allowed to proceed overnight at
room temperature. The reaction yield was more than 95 % for
35   all three reactions. The respective reaction solutions of
Conjugates I and II were adjusted with 10 M sodium
hydroxide to pH 10, and 100 µl of acetic anhydride were
then added under vigorous stirring in order to acetylate

the remaining amino groups. The heparin conjugates
obtained, Conjugate I, Conjugate II and Conjugate III,
respectively, were purified on a Sephacryl® S-400 column
(Pharmacia LKB Biotechnology AB, Sweden), the conjugates
being obtained in the void fraction.

Tubings of polyethylene were prepared with the three
obtained heparin conjugates for the thrombin test according
to Example 1, the following results being obtained.

	Uptake of thrombin (without defib. plasma)	Res. amount of thrombin (with defib. plasma)
Conjugate I	0.437 ± 0.008	0.007 ± 0.002
Conjugate II	0.445 ± 0.020	0.003 ± 0.001
Conjugate III	0.501 ± 0.032	0.005 ± 0.002

The results demonstrate that all three conjugates give
a perfectly satisfactory effect.

EXAMPLE 8
Preparation of surfaces with different amino-functional substrate surfaces

Tubings of polyethylene were heparinized as follows,
the added designations A, B, C and D, respectively,
indicating alternative amino-functionalizing treatments of
the tubing surface:

1.   Ammonium persulphate (1 %, 60°C, 60 min.)

2A.   Polyethylenimine (0.3 mg/ml, borate pH 9, RT, 15 min.)

2B.   Polyallylamine (10 mg/ml, borate pH 9, RT, 15 min.)

2C.   Chitosan (10 mg/ml, 1 % HAc, RT, 15 min.)

2D.   Polylysine (5 mg/ml in water, RT, 15 min.)

3.   Heparin-polylysine conjugate prepared according to
Example 1 (50 μg/ml in citrate buffer, 0.5 M NaCl, pH 3.8,
RT, 120 min.).

The surfaces thus prepared were thoroughly rinsed with
borate buffer, pH 9, and water.

Polyethylene tubings heparinized according to the
above four alternatives were tested with regard to uptake
and inhibition of thrombin, as described in Example 1, all
the alternatives exhibiting a perfectly satisfactory
effect.

EXAMPLE 9

Surface-heparinization of a lens (PMMA) and test of platelet adhesion

Intraocular lenses of polymethyl methacrylate (PMMA)
5   were heparinized according to Example 1 and then tested
with regard to adhesion of platelets.

Non-modified and surface-heparinized lenses,
respectively, were incubated in fresh human citrated whole
blood under constant motion for 60 minutes. The lenses were
10 then rinsed repeatedly in sodium chloride solution to
remove all adhering blood. Finally, adenosine triphosphate
(ATP) was extracted from any platelets which had adhered to
the lens surface, and the content of ATP obtained was
determined by bioluminescence. Platelet adhesion to the
15 heparinized lenses was reduced with 98 % compared with the
untreated control lenses.

EXAMPLE 10

Adsorption of heparin conjugate to a " biological surface"

In order to examine whether a heparin conjugate
20 prepared according to the present invention can be adsorbed
irreversibly to a surface coated with thrombotic biological
material the following experiments were carried out:

Non-surface-modified tubings of polyethylene were
half-filled with citrated whole blood and rotated for 60
25 minutes. The tubings were then drained of blood and
thoroughly rinsed with sodium chloride solution. Hereby the
tubings were coated with thrombotic material consisting of
plasma proteins and platelets in different activation
stages. Heparin-polylysine conjugate prepared according to
30 Example 1 was diluted to a final concentration of 100 μg/ml
in sodium chloride solution, which solution then was
rotated in the tubings for 60 minutes. The tubings were
finally thoroughly rinsed with borate buffer, pH 9, and
water.

35   The tubings thus heparinized were tested with regard
to uptake and inhibition of thrombin according to
Example 1, the test tubings exhibiting a perfectly
satisfactory effect.

EXAMPLE 11
Combination preparation with urease

Polylysine (10 mg, molecular weight 464,000) was
dissolved in 1.5 ml water, to which 1.0 ml of SPDP
(10 mg/ml MeOH) was added under shaking, and the reaction
was then allowed to proceed for 30 minutes. The sample was
loaded on a PD-10 column and eluted with 0.9 % NaCl. The
void fraction was collected, and analysis indicated that
the content of SPDP was 1.053 μmole/ml.

Urease (U-1500, from jackbean, Sigma, USA) was
dissolved to 10 mg/ml in phosphate buffer, pH 7.5, and
filtrated through a 0.22 μm filter. The content of free SH-
groups was determined as 0.161 μmole/ml.

Polylysine-SPDP dissolved in 3 M NaCl was mixed with
urease, such that about 10 % of available SPDP-groups could
undergo disulphide exchange with SH-groups of the urease.
Spectrophotometrical measurement at 343 nm confirmed that
this had taken place. Then, heparin modified with free SH-
groups according to Example 1 was added, the amount of
added heparin-SH corresponding to the remaining 90 % of
available SPDP-groups. The reaction proceeded to com-
pletion. The obtained conjugate was finally purified on a
Sephacryl® S-400 column (Pharmacia LKB Biotechnology AB,
Sweden), the conjugate being obtained in the void fraction.
Testing of the obtained conjugate indicated that heparin
activity as well as urease activity could be detected.

CLAIMS

1.   A substantially water-soluble biologically active
conjugate comprising a substantially straight-chained
organic polymer having a number of functional groups
distributed along the polymer backbone chain, via which
groups at least about 20 molecules from the group of
sulphated glycosaminoglycans in a non-active part thereof
are anchored through covalent bonds.

2.   A conjugate according to Claim 1, characterized in
that said polymer is derived from a natural or a synthetic
polypeptide, polysaccharide or aliphatic polymer.

3.   A conjugate according to Claim 2, characterized in
that said polymer chain is derived from polylysine, poly-
ornithine, chitosan, polyimine or polyallylamine.

4.   A conjugate according to Claim 1, 2 or 3,
characterized in that the glycosaminoglycans substantially
are bound to the polymer backbone chain via a single bond,
preferably terminally.

5.   A conjugate according to any one of Claims 1 to 4,
characterized in that the glycosaminoglycans are bound to
the polymer backbone chain via an amino group associated
with the glycosaminoglycans.

6.   A conjugate according to any one of Claims 1 to 5,
characterized in that the conjugate due to the glycos-
aminoglycans has a sufficient polyanionic character when
dissolved in water to be capable of being bound
substantially irreversibly along substantially the whole of
its length to a positively charged substrate surface
through electrostatic interaction.

7.   A conjugate according to any one of Claims 1 to 6,
characterized in that it has at least 30 glycosaminoglycan
residues.

5   8.   A conjugate according to Claim 7, characterized in
that it has at least 100 glycosaminoglycan residues.

9.   A conjugate according to any one of Claims 1 to 8,
characterized in that said glycosaminoglycan is heparin or
10   a fragment or derivative thereof.

10.   A conjugate according to any one of Claims 1 to 9,
characterized in that the glycosaminoglycan residues are
bound to the polymer backbone chain via a coupling
15   sequence.

11.   A conjugate according to Claim 10, characterized in
that said coupling sequence is derived from a hetero-
bifunctional coupling reagent.
20

12   A conjugate according to any one of Claims 1 to 11,
characterized in that the polymer backbone chain in
addition to the glycosaminoglycans supports residues of at
least one additional, biologically active substance.
25

13.   A prepared substrate surface comprising a biologically
active conjugate affinity-bound to the surface, charac-
terized in that the conjugate comprises a substantially
straight-chained organic polymer having a number of
30   functional groups distributed along the polymer backbone
chain, via which groups a number of molecules from the
group of sulphated glycosaminoglycans are anchored through
covalent bonds, the conjugate preferably being bound to the
surface through electrostatic interaction between the
35   conjugate and the substrate surface.

14. A prepared substrate surface according to Claim 13,
characterized in that the biologically active conjugate is
a conjugate according to any one of Claims 1 to 11.

5   15. A process of preparing a biologically active conjugate
comprising a substantially straight-chained organic polymer
supporting a number of molecules from the group of
sulphated glycosaminoglycans, characterized in that the
process comprises providing a substantially straight-
10 chained organic polymer having a number of functional
groups distributed along the polymer backbone chain, and to
these functional groups, optionally via a coupling reagent,
covalently binding a number of molecules from the group of
sulphated glycosaminoglycans in a non-active part thereof.

15

16. A process of preparing a surface with sulphated
glycosaminoglycans, characterized by contacting a conjugate
comprising a substantially straight-chained organic polymer
having a number of functional groups distributed along the
20 polymer backbone chain, via which groups a number of
molecules from the group of sulphated glycosaminoglycans
are anchored through covalent bonds, with a substrate
surface having affinity to the conjugate, such that the
conjugate is bound substantially irreversibly thereto.

25

17. A process according to Claim 16, characterized in that
the conjugate has polyanionic character and that the
substrate surface is cationic.

30 18. A biologically active conjugate according to any one
of Claims 1 to 12 for use as a therapeutical agent.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 31/725, 47/48, A 61 L 33/00, C 08 B 37/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category*	Citation of Document,[11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	EP, A1, 0294905 (SENTRON V.O.F.) 14 December 1988, see the whole document	1-3
X	US, A, 4415490 (YASUSHI JOH) 15 November 1983, see the whole document	1-3
A	WO, A1, 8700060 (BATTELLE MEMORIAL INSTITUTE) 15 January 1987, see the whole document	1-17
A	EP, A1, 0212933 (KOKEN CO. LTD) 4 March 1987, see the whole document	1-17

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23rd December 1992	29 -12- 1992

International Searching Authority	Signature of Authorized Officer
SWEDISH PATENT OFFICE	Anneli Jönsson

Form PCT/ISA/210 (second sheet) (January 1985)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on   02/12/92
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